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Morphological and molecular identification of potato cyst-forming nematode *Globodera pallida* in soil samples from Costa Rica

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ABSTRACT

Morphological and molecular analyses were used to identify the potato cyst-forming nematodes found in soil samples from Costa Rica. The morphometric characters analyzed in cysts and second-stage juveniles conformed to those of *Globodera pallida*. PCR with species-specific primers (single or multiplex reaction) produced an amplification product of the same size as the *G. pallida* control one. The PCR-RFLP patterns obtained from the Costa Rican samples revealed no difference among them and were identical to those of the *G. pallida* control. Evidence of the presence of *G. rostochiensis* was not found. This is the first molecular report of the presence of *G. pallida* in Costa Rica.

Key words: *Globodera rostochiensis,* PCR, PCR-RFLP, ITS region.

Identificación morfológica y molecular del nematodo formador de quistes *Globodera pallida* en muestras de suelo de Costa Rica

RESUMEN

Se realizaron análisis morfológicos y moleculares para la identificación de nematodos formadores de quiste de la papa en muestras de suelo de Costa Rica. Los caracteres morfométricos evaluados en quistes e individuos en segundo estado juvenil, correspondieron a los de *Globodera pallida*. Los PCR llevados a cabo con iniciadores especie-específicos (solos o en reacción múltiplex) produjeron un producto de amplificación del mismo tamaño que el producido para el control de *G. pallida*. Los patrones PCR-RFLP obtenidos de las muestras de Costa Rica no mostraron ninguna diferencia entre ellos y resultaron idénticos a los del control *G. pallida*. No se encontró evidencia de la presencia de *G. rostochiensis* en las muestras analizadas. Este es el primer reporte molecular de la presencia de *G. pallida* en Costa Rica.

Key words: *Globodera rostochiensis,* PCR-RFLP, región ITS.

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Introduction

Potato (*Solanum tuberosum*) is an important staple crop in Costa Rica, ranking third after rice and dry beans in the national diet, with a *per capita* consumption of 25 kg *per annum*. Approximately 3000 ha of potato crops are grown annually in the major production regions of Cartago and Zarcero (Brenes *et al*., 2002).

Potato cyst nematodes (PCN), namely *Globodera pallida* (Stone) Behrens (white potato nematode) and *G. rostochiensis* (Woll.) Behrens (yellow or golden potato nematode), cause substantial damage to potato crops worldwide, both species having a quarantine status (OEPP/EPPO, 2004). The centre of origin of the two species is in the Andean mountains of South America from where they were taken to other parts of the world, possibly together with potatoes. The present distribution covers sea level areas in temperate zones and high tropical altitudes, always linked to that of potato crops. In Latin America PCNs are the most common nematode parasites of this crop, and other solanaceous hosts (Sullivan *et al*., 2007). Up to date, they are known to be present in most of the South American countries, and in Panama and Costa Rica in Central America. In North America PCNs have been found in Canada (Newfoundland, British Columbia and Vancouver Island only), Mexico and USA (CABI/EPPO, 2007; Sullivan *et al*., 2007). Although PCN infestations do not produce obvious symptoms, they can damage the roots and reduce yield. When infestations are severe, roots are more seriously damaged and may be killed (CSL, 2003). Damage may also appear as signs of mineral deficiency, as patches of stunted yellowing plants, or as wilting due to an inefficient root system, but such symptoms usually show up only when infestation levels are already high. Several other factors such as differences in yield potential between sites, cultivar tolerance to damage, differences in crop management, weather, etc., may increase or decrease nematode damage (Hockland, 2002).

Depending on the severity of the damage, PCNs can reduce harvest to a highly considerable extent, which often depends on the variety grown and on farming practices. In Mexico, for example, annual losses caused by *G. rostochiensis* in potato (Wollenberg) have been estimated to reach up to 70% (Tovar *et al*., 2006).

G. rostochiensis was first reported for Costa Rica in 1973 (Ramírez, 1979). However, successive surveys (1975-77, 1981, 1984-85, 1988, 1995-97, 1998) carried out by local officials in collaboration with international experts in many parts of the country, including places where the nematode had first been found, failed to confirm its presence (Brodie, 1998; OIRSA, 1999). In 1999, the European and Mediterranean Plant Protection Organization (EPPO) indicated that *G. rostochiensis* should be considered as "Absent: pest no longer present" in Costa Rica (OIRSA, 1999). Since then, *G. pallida* had not been detected in the country until the current report. At the beginning of 2005, some potato fields in the region of Cartago, the most important potato production area of the country, showed unusual symptoms. Soil and plant samples taken from those fields revealed the presence of cyst-forming nematodes, thus raising questions about their identity.

PCN identification is based on the examination of morphological features. However, both species are morphologically and morphometrically closely related. Morphological identification demands considerable skills and is time-consuming (OEPP/EPPO, 2004). Molecular analysis of DNA by polymerase chain reaction (PCR) offers an alternative method for the diagnosis of nematodes, including PCNs (Powers, 2004). Several PCR tests have been developed to distinguish the two PCN species, including PCR-RFLP (restriction fragment length polymorphisms) and PCR with species-specific primers used in single or multiplex reactions (Bulman and Marshall, 1997; Blok *et al*., 1998; Fullaondo *et al*., 1999; Vejl *et al*., 2002)

The objective of the present study was then to identify the PCNs recently found in soil samples from Costa Rica, by means of morphology and PCR-based methods.

Materials and methods

Nematode collection. Soil and plant samples were collected from two farms in northern Cartago and brought to the Nematology Laboratory of the University of Costa Rica. Cysts were extracted using a Fenwick can (Fenwick, 1940) and then air dried, to be finally placed into 1.5 mL tubes and stored at room temperature.

Morphological identification. After recording the color of the females present in the samples, morphometric data were taken from 50 cysts and 50 second-stage juveniles. In analyzing the cysts, the number of cuticular ridges between vulva and anus, the distance from the anus to the nearest edge of the fenestra, and the length of the fenestra were determined. The two latter measures were used to calculate Granek´s ratio. In the juveniles, stylet length and stylet knob shape were determined. All measurements and observations were taken using an Olympus BH-2 microscope. Range and average of all measurements were calculated. The juveniles were heat fixed before measuring.

DNA extraction. Genomic DNA was extracted from samples of 10 cysts each, using a CTAB extraction procedure. DNA was extracted twice with chloroform-isoamyl alcohol (24:1), and precipitated by adding 3M sodium acetate and isopropanol. Quantity and quality were evaluated through UV illumination and ethidium bromide staining on 1% agarose gel. A total of 10 samples from each farm were analyzed.

PCR with species-specific primers. Single and multiplex PCR reactions using species-specific primers were performed to distinguish between *G. rostochiensis* and *G. pallida.* RAPD-based specific primer sets GroF/GroR (for *G. rostochiensis*, amplicon size 315 bp) and GpaF/GpaR (for *G. pallida*, amplicon size 798 bp) (Fullaondo *et al*., 1999) were used in single PCR reactions. Primers PITSr3 (for *G. rostochiensis*, amplicon size 434 bp) and PITSp4 (for *G. pallida*, amplicon size 265 bp) (Bulman and Marshall, 1997) were used in combination with common primer ITS5 (White *et al*., 1990) in a multiplex reaction. DNA samples from *G. rostochiensis*, *G. pallida*, and from a mixture of Spanish species kindly provided by Dr. E. Ritter (Basque Institute for Agricultural Research and Development (NEIKER), Spain), were used as positive controls.

PCR-RFLP. The internal transcribed spacer region (ITS) of the ribosomal DNA repeat unit was amplified by PCR using primer pair ITS4 - ITS6 (White *et. al.*, 1990). Ten microliters of the PCR product were digested by one of the following restriction enzymes: *Alu*I, *Msp*I and *Rsa*I, contained in the buffer supplied by the manufacturer. Digestion was allowed to proceed at 37 °C for 4h.

PCR conditions. PCR reactions were carried out in a total volume of 25 µl containing 4 µl of nematode DNA, 2.5 µl of 10X PCR buffer, 2.0 mM $MgCl_2$, 0.1 µM of each dNTP, 0.4 µM of each primer, and 1 unit of Taq DNA polymerase (FERMENTAS, USA). Reaction steps consisted of an initial denaturation at 94 °C for 3 min, followed by 35 (specific primers) or 25 (ITS region) denaturation cycles at 94 °C for 45 s, annealing at 60 °C (specific primers) or at 57 °C (ITS region) for 1 min, an extension cycle at 72 °C for 45 s, and a final one at 72 °C for 5 min. Possible contamination was checked by including negative controls (no DNA) in all amplifications. The amplified fragments were separated by electrophoresis on 1.6% agarose gels. The restricted products were separated on 2% agarose. All products were visualized with UV illumination (Cole Palmer System, Illinois, USA).

Results

Morphological identification. Female color is used as a species indicator in potato cyst nematode sample analysis. *G. rostochiensis* females change from white to yellow, and then to a brown cyst (chromogenesis), while *G. pallida* ones change directly from white to brown (OEPP/EPPO, 2004). The females in the studied Costa Rican soil samples were observed to be white. No yellow females were seen, even in samples corresponding to different dates. Brown cysts were always present in the samples.

Table 1 shows the ranges and averages of the measurements taken from the studied cysts and second-stage juveniles as compared to the *G. rostochiensis* and *G. pallida* standards. Stylet shape and length of the heat fixed juveniles taken from the samples conformed to those of *G. pallida*. So did the range and average of the number of cuticular ridges between anus and fenestra. Granek´s ratio (the distance from the anus to the nearest edge of the fenestra, divided by the length of the fenestra) averaged 2.1. Lower than 3 mean Granek´s ratio values are associated to *G. pallida*, whereas values above 3 correspond to *G. rostochiensis*.

Table 1. Range and mean values of measurements of *Globodera rostochiensis*, *Globodera pallida* and the Costa Rican samples

Species/Sample	Shape of anterior surface of knob	J2 stylet length (µm)	Number of cuticula ridges between vulval basin and anus	Granek's ratio
*Globodera rostochiensis**	rounded	(21.8)	16-31 (>14)	1.3-9.5 (>3)
*Globodera pallida**	pointed	22-24 (23.8)	8-20 (<14)	1.2-3.5 (<3)
Costa Rican potato cyst nematode**	pointed	8.8 (24.2)	6-14 (9.4)	1.9-2.4 (2.1)

*Taken from OEPP/EPPO. 2004. OEPP/EPPO Bulletin 34: 155-167.
** Measurements taken from 50 cysts and 50 second-stage juveniles.

PCR species-specific primers. All DNA extracts from the Costa Rican samples produced amplification products of the same size as those obtained from the *G. pallida* control, when species-specific primers were used in single (Fig. 1) or multiplex (Fig. 2) PCR reactions. Amplification products of the same size as those reported for *G. rostochiensis* were only obtained from the *G. rostochiensis* control and from the Spanish species mixture (Figs. 1B; 2).

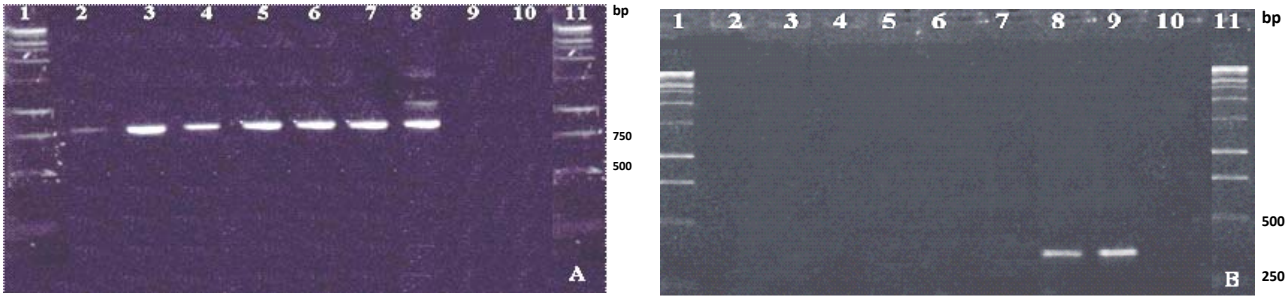

Figure 1. Molecular Identification of potato cyst-forming nematodes from Costa Rica with specific primers. A) *G. pallida* specific primers GpaF and GpaR. B) *G. rostochiensis* specific primers GroF and GroR. Lane 1, Molecular weight marker (M)= 1 kb (Fermentas); Lane 2, QLC11; Lane, QLC50a; Lane 4, QLC50b; Lane 5, QLC100; Lane 6, QPC100; Lane 7, *G. pallida*; Lane 8, *G. pallida* + *G. rostochiensis*; Lane 9, *G. rostochiensis;* Lane 10, Negative control; Lane 11, M= 1kb.

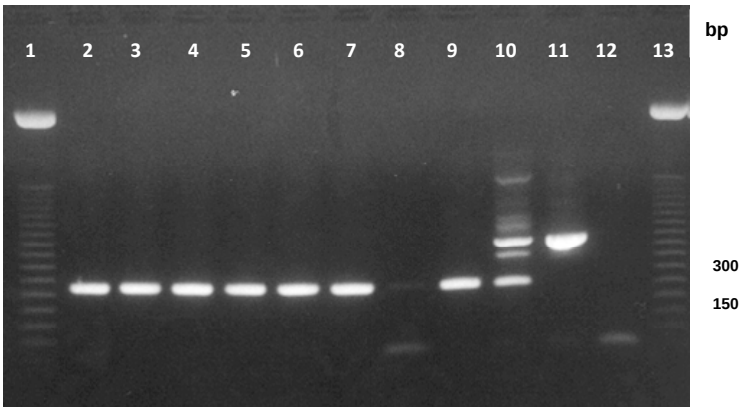

Figure 2. Molecular identification of Costa Rican PCN. Multiplex polymerase chain reaction using primers PITSr3, PITSp4 and the common primer ITS5: Lane 1, Molecular weight markers (M)=50 bp DNA ladder (FERMENTAS); Lane 2-8, PCN from Costa Rica; Lane 9, *G. pallida* from Spain; Lane 10, *G. pallida* + *G. rostochiensis* mixture from Spain; Lane 11, *G. rostochiensis* from Spain; Lane 12, no DNA; Lane 13, M=50 bp.

PCR-RFLP. Amplification of the ITS region yielded a single fragment of approximately 1 kb for all samples, including the reference populations. The three restriction enzymes generated RFLPs that allowed identifying the two *Globodera* species (Fig. 3). The RFLP patterns obtained from the Costa Rican samples revealed no difference among them and were identical to those of *G. pallida* (Fig. 3).

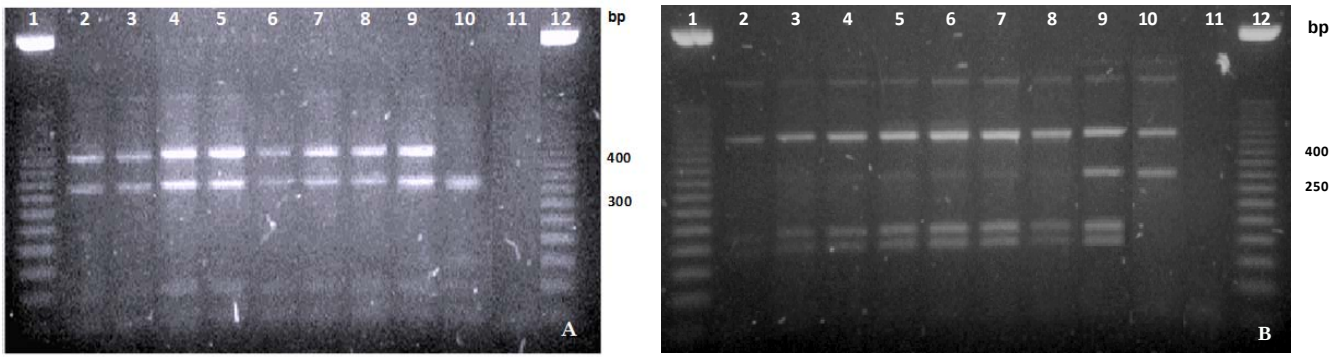

Figure 3. Molecular identification of Costa Rican PCN. Restriction fragments of amplified ITS regions digested by A) *Alu I* and B) *Rsa* I: Lane 1, Molecular weight markers (M)=50 bp DNA ladder (FERMENTAS); Lane 2-7, PCN from Costa Rica; Lane 8, *G. pallida* from Spain; Lane 9, *G. rostochiensis* from Spain; Lane 10, *G. pallida* + *G. rostochiensis* ; Lane 11, no DNA; Lane 12, M=50 bp.

Discussion

The correct identification of a species should be based on the congruence of several methods. Morphological and molecular methods enable the rapid and reliable identification of different species. For quarantine species such as PCNs, it is recommended to combine both methods, plus additional criteria if available. In the present study, morphological and molecular methods were used for the identification of a PCN population present in soil samples from Costa Rica. Both methods identified the parasite as *Globodera pallida*. To the best of our knowledge, this is the first report of this species

for potato crops in Costa Rica. Granek´s ratio is considered to be the most informative nematode morphometric measurement currently available, particularly for separating *G. pallida* and *G. rostochiensis* (Fleming and Powers, 1998). The lower limit of the Costa Rican sample Granek´s ratio values was slightly higher than the *G. pallida* standard (Table 1). This might be explained by the fact that fenestra length, which is shorter than vulval basin diameter, was used instead of the latter to calculate Granek´s ratio, thus resulting in higher a Granek´s ratio value.

The origin of the presence of *G. pallida* in Costa Rica is currently unknown. Both PCN species, *G. rostochiensis* and *G. pallida,* are present in Panama the neighboring country (Tarte, 1968; Brodie, 1998), and in the rest of the countries of Latin America (Sullivan *et al*., 2007). Hence, introduction from those countries may have occurred, perhaps in an unofficial potato shipment. Comparative molecular analyses of *G. pallida* populations from both regions might help elucidate the source of the introduction.

The time of such introduction is also unknown. It is likely to have happened after the last *G. rostochiensis* survey, carried out in 1998 (OIRSA, 1999), which covered the same areas where *G. pallida* is currently present. Since both species are morphologically related and probably share similar niches, the absence of *G. rostochiensis* could indicate that *G. pallida* was also absent at that time. The lag between introduction and detection could be explained by the fact that, under field conditions, the introduction of PCNs can remain unnoticed for several years, only reaching detectable sample levels after six years (Spears, 1968).

No evidence of the occurrence of *G. rostochiensis* in Costa Rica was found, which supports the recent declaration by the European and Mediterranean Plant Protection Organization (EPPO), indicating that *G. rostochiensis* can be considered as “Absent: pest no longer present” in Costa Rica (OIRSA, 1999).

The presence of *G. pallida* in Costa Rica represents a threat on local potato cultivation, not only because of its direct impact on crop yield, but also because it limits exportation to PCN free countries (Sullivan *et al*., 2007).

Measures must be taken in order to prevent the spread of potato nematodes into new areas, and to keep nematode density below damage levels in infested areas. Early detection of infestations, integrated nematode management through quarantine regulations, sanitary principles, crop rotation and physical and chemical control and use of resistant varieties may help reduce the nematode’s population density to levels that allow successful and profitable potato production (Franco, 1986; Trudgill, 1986; Elston *et al*., 1991). Trap cropping could be an alternative control method for PCNs, in as much as it stimulates juveniles to hatch from cysts and invade its roots, to be destroyed before the nematodes complete their life cycle, thus reducing soil infestation (Scholte, 2000).

The PCR-based methods used in the present study shall play an important role for future surveys and for routine identification of *G. pallida*, in order to contain the nematode to its current distribution area. In addition, the use of species-specific primers in PCR multiplex reactions, which allows the simultaneous identification of both species in the same sample, will eventually help detecting *G. rostochiensis*.

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